Conclusions: In our experimental conditions, the capability to induce chondrogenic differentiation depends on the culture conditions used. The transcription factor that actuates better in normoxic condition differs from the one that displays a better action in a hypoxic atmosphere.
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GENE EXPRESSION CHANGES ASSOCIATED WITH CHONDROGENIC DIFFERENTIATION OF HUMAN MSCS

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Purpose: Human mesenchymal stem cells (hMSCs) are useful for cartilage tissue engineering as they can differentiate into chondrocyte-like cells under conditions including high cell density and a defined medium. Cartilage markers become detectable at the RNA and protein levels within the first week of differentiation. The phenotypic changes are the result of a reprogramming of the gene expression and physiology of MSCs. To support our studies of chondrogenic MSC differentiation, we used gene expression microarrays to understand the sequence and extent of these changes.
Methods: Five hMSC preparations were derived from bone marrow aspirates obtained under an IRB-approved protocol. The cells were expanded through the end of first passage in DMEM with serum and 10 ng/ml FGF-2, then moved to aggregate culture in chondrogenic medium to induce differentiation. Total RNA was extracted from 20 aggregates per prep at days 0, 1, 3, 5, and 7; 5-8 μg of RNA were reverse-transcribed. The three best cDNA preps were analyzed using Affymetrix U133A 2.0 microarrays. Spiked bacterial IVT were used as hybridization controls. Raw data generated using GCOS software were scaled to 500 and imported into Agilent GeneSpring 6.4. A short list of interesting genes was generated by binary comparisons at each time point using MAS5.0 algorithms in GCOS. Transcripts consistently called as increased or decreased in all three preparations with an average absolute fold change $\geq$ 2.0 were included. For genes referenced by multiple probesets, consistency checks were performed, and we used the median value of the probesets. Filtered data were then analyzed using GeneSpring, Ariadne Genomics Pathway Studio and Partek Genomics Suite. Cluster analysis and principal component analysis were performed on the filtered data. A 3 $\times$ 3 array of self-organizing maps (SOM) was created, and regulation pattern detection was performed; finally assignment to specific cellular pathways attempted. We also mined the database for chondrogenesis-related genes known to be modulated during MSC differentiation from our experiments or from published reports.
Results: All arrays passed internal quality control. After filtering the data, we found a high-value population of 3,285 differentially expressed genes. Cluster and SOM analysis revealed that most of the changes occurred between days 0 and 1 (1,723) or 2, with fewer changes occurring later. Small subsets spiked or plummeted transiently on day one, even smaller subsets showed steady in- or decreases over the study period. Pathway assignment performed on the SOM groups suggests that genes regulating transcription, cell-cycle, cell adhesion, and differentiation are highly regulated (both up and down) beginning at the earliest time points.
Conclusions: We have a stringent database of gene expression changes during early chondrogenic differentiation of hMSCs. Pattern analyses indicate that most changes occur within the first day and are permanent; this suggests genes turned on or off during differentiation that are, thus, MSC- or chondrocyte-specific. A much smaller set was transient, suggesting involvement in gene expression regulation and lineage commitment. The number of regulated genes affecting transcription, cell-cycle, cell adhesion, and differentiation reflects the significant reprogramming of the cells in the transition from proliferation to differentiation. The database will be a useful tool for interpreting molecular changes associated with chondrogenic differentiation of MSCs.

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EFFECT OF FIBRONECTIN SPLICING ON CELL PROLIFERATION AND CHONDROCYTE-SPECIFIC GENE EXPRESSION IN A MESENCHYMAL STEM CELL SYSTEM

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Purpose: During embryonic development, expression of fibronectins (FNs) bearing the alternatively spliced EIIIA and V segments is prevalent in prechondrogenic mesenchymal cell condensations. However, expression of these FNs becomes restricted near the time of chondrogenesis, suggesting that they may not be compatible with processes by which mesenchymal stem cells (MSCs) differentiate to chondrocytes.
Methods: We tested the effects of the EIIIA and V segments, expressed as glutathione S-transferase (GST) fusion proteins, adsorbed to plastic or in solution, on proliferation and gene expression in rat RCJ 3.1 multipotential mesenchymal cells, an MSC model cell line, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and real-time PCR. A GST fusion protein containing the 10th type III repeat of FN (III-10) and poly-L-lysine (PLL) were also tested.
Results: In 15% fetal bovine serum (FBS), cells grown in monolayer for 7 days (d) on surfaces coated with EIIIA, V, III-10, or PLL appeared confluent, and attained similar MTT scores, with $OD_{570-630}$ values (average$\pm$S.D.) of 1.3$\pm$0.8, 1.4$\pm$0.8, 1.3$\pm$0.7, and 1.5$\pm$1.0, respectively (ns, N=3). Under serum-free conditions, more cells remained adherent to surfaces coated with III-10 than the other 3 agents after 7 d in HEScGRO (Millipore), with respective values of 0.2$\pm$0.1**, 0.2$\pm$0.1**, 0.6$\pm$0.2, and 0.4$\pm$0.2*. In KnockoutSR (GIBCO), V and III-10 both promoted greater retention of adherent cells than EIIIA and PLL, with respective values for the 4 agents of 0.2$\pm$0.0***, 0.6$\pm$0.1, 0.7$\pm$0.1, and 0.3$\pm$0.1**. Aggrecan expression in cells grown in 15% FBS for 7 d on each of the substrates decreased compared to cells grown for 1 d on III-10, but not significantly so (fold changes of -1.0$\pm$0.7, -0.7$\pm$0.6, -2.8$\pm$0.1, and -2.6$\pm$0.3, respectively) (N=3). In contrast, aggrecan expression increased in cells grown in pellet culture in the presence of TGF-b, with a fold change of 2.5$\pm$1.0 at 14 d compared to cells at 0 d (ns, N=3). Addition of soluble EIIIA (200 nM) to such cultures triggered reduced aggrecan expression so that the fold change at 14 d compared to 0 d cells was -7.1$\pm$0.1 (18.6-fold reduction compared to untreated cells, ***, N=3). p>0.05=ns, p<0.05=* o<0.01=***, p<0.001=***
Conclusions: The 10th type III repeat and V segment of FN promote anchorage-dependent proliferation/survival of RCJ 3.1 MSCs in serum-free culture. These cells lose aggrecan expression in monolayer culture, and this is not appreciably affected by apposition to EIIIA, V, or III-10. In contrast, aggrecan expression increases during pellet culture, and such acquisition of chondrocyte-specific gene expression is inhibited by soluble EIIIA, suggesting that this FN segment could potentially constitute a therapeutic target in efforts to promote chondrocytic differentiation of MSCs, or to reverse cartilage degeneration in osteoarthritis. Research funded by a VA Merit grant.

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SEQUENTIALLY PROGRAMMED MAGNETIC FIELD THERAPY AS AN EFFECTIVE TREATMENT FOR OSTEOARTHRITIS

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Introduction and Purpose: Different modalities of available treatment for treating osteoarthritis, including newer drugs like NSAIDS, Intra-articular injections, etc have been tried with very little or no success, leaving patients with the only surgical option of joint replacement. All of these except surgery provide only symptomatic relief and do not reverse the continuous damage done to the cartilage. The newer drugs also come withmany undesirable side effects. Total knee replacement Surgery (TKR) is often beyond the financial reach of many patients, especially in developing countries. Even TKR surgery has a severe painful recovery period and a low patient acceptance. So there has always been a need for a safe treatment method which would be pain free and also reverse the disease process.
Sequentially Programmed Magnetic Field (SPMF) Therapy utilizes Magnetic Field Generators (MFG) that can be precisely controlled and focused onto the affected tissues. The normal physiological remodeling of cartilage occurs due to piezoelectric stimuli which are lost in osteoarthritic patients.

By utilizing specific frequencies in the range of 6-30Hz based on tissue type and grade of osteoarthritis this stimulus is recreated by SPMF therapy leading to cartilage regeneration.
This recreated peizoelectric stimuli, is known to enhance mitosis and cell regeneration by normalizing the abberant electromagnetic fields of microtubules of the centrioles
Methods: 195 patients with bilateral osteoarthritis (OA) of knees were assessed by internationally recognized Knee Society clinical rating system; the scores computed prior to treatment, after 21 days of therapy, at 3 and 9 months. In addition, MRI of the treated knees was done using standard protocol, before treatment, at three and nine months after treatment, to measure objective changes in cartilage thickness.
Results: This non-randomized, phase II study showed statistically highly significant improvement in pain scores, total knee scores, total functional scores and the range of motion, immediately after the treatment vis-à-vis pre-treatment values, and this improvement persisted when re-evaluated at three and nine months. There was also a significant increase in cartilage thickness at three months and nine months from 0.64mm (±0.02) baseline to 0.88 mm (±0.07) in left knee, and 0.65mm (±0.02) to 0.89mm (±0.05) in the right knee joint ($p<0.001$) at 3 months and to 1.26mm (±0.02mm) in the left knee and 1.23mm (±0.03mm) in the right knee at the end of 9 months.
Conclusions: Therapeutic exposure to SPMF therapy is effective in ameliorating the signs and symptoms of OA, and inducing regeneration of chondrocytes as evidenced by increase in cartilage thickness. SPMF therapy is an effective treatment modality for osteoarthritis.

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EXPANSION AND CHONDROGENIC DIFFERENTIATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOLLOWING RAAV-MEDIATED GENE TRANSFER AND OVEREXPRESSION OF HUMAN FGF-2

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Purpose: Articular cartilage lesions are an important problem in orthopaedic surgery. The use of progenitor cells such as bone marrow-derived mesenchymal stem cells (MSCs), as platforms to deliver candidate genes in a suitable conformation is an attractive approach to enhance cartilage repair. Here, we tested the ability of rAAV vectors to overexpress FGF-2 that promotes the expansion and differentiation of human MSCs in a three-dimensional environment.
Methods: rAAV were packaged, purified, and titrated as previously described. All transgene cassettes were controlled by the CMV-IE promoter/enhancer: *E. coli* beta-galactosidase (rAAV-*lacZ*), Red Fluorescent Protein (rAAV-RFP), Firefly luciferase (rAAV-*luc*), and a human basic fibroblast growth factor (hFGF-2) cDNA (rAAV-hFGF-2). Bone marrow aspirates were obtained from the iliac crest of donors. Aspirates were layered onto Histopaque-1077 density gradient and the nucleated cell fraction was collected and resuspended in hMSC culture medium containing stimulatory supplements (StemCell Technologies Inc.). Cells were transduced with rAAV for up to 21 days in monolayer or in aggregate cultures (2×10^5 cells). Aggregates were kept in DMEM high glucose, ITS$^+$ Premix, pyruvate, ascorbate 2-phosphate, dexamethasone, and TGF-β. Transgene expression was determined by X-Gal staining, live fluorescence, luciferase assay, FGF-2 ELISA, and by immunocyto- and immunohistochemistry. Paraffin-embedded sections of aggregates (5 μm) were stained with toluidine blue and alizarin red. Indirect immunostaining was also performed to detect type-II and type-X collagen, Runx-2, and Sox9. The DNA contents were monitored by fluorimetric assay, the proteoglycan contents by binding to DMMB, and the type-II collagen contents by ELISA. Morphometric measurements were performed at 3 standardized sites by image analysis. Each condition was performed in triplicate in 2 independent experiments. Data are expressed as mean ± SD. The t-test was employed with $P<0.05$ considered statistically significant.
Results: Sustained, dose-dependent transgene expression was observed in monolayer cultures of hMSCs, with FGF-2 levels being from 350.2±2.5 to 41.1±1.2 pg/ml/24 h over time in rAAV-hFGF-2-transduced cells vis a vis rAAV-*lacZ* (< 0.01 pg/ml/24 h). Luciferase activity was of 16.3±0.6 RLU/mg total proteins after 21 days in rAAV-*luc*-transduced cells compared with rAAV-*lacZ* (0.2±0.1 RLU/mg total proteins). Efficient, sustained transgene expression was also seen in aggregates (Fig. 1), with FGF-2 levels from 375.1±1.6 to 136.1±1.4 pg/ml/24 h over time in rAAV-hFGF-2 aggregates

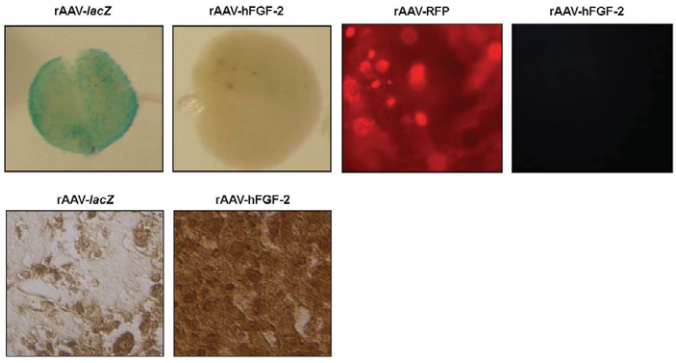

Figure 1. Transgene expression in hMSCs aggregates (day 21).

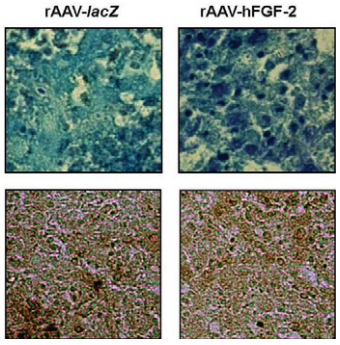

Figure 2. Toluidine blue staining and detection of type-II collagen in hMSCs aggregates (day 21).

vis a vis rAAV-*lacZ* (1.51±0.4 pg/ml/24 h). Chondrogenic differentiation of cells was observed after 21 days in all types of aggregates (Fig. 2). Sox9 immunoreactivity was visible, whereas that for type-X collagen and Runx-2 and alizarin red staining were less intense. The diameters (856±30 versus 538±36 μm), DNA contents (1.039±0.005 versus 0.942±0.009 ng/mg total proteins), and cell numbers (862±14 versus 435±12 cells/mm^2) of the FGF-2 aggregates were significantly higher than those of the *lacZ* aggregates (1.6-fold, 1.2-fold, and 1.9-fold, respectively; always $P<0.001$). In contrast, there was no difference in the proteoglycan contents (0.80±0.10 vs. 0.77±0.05 ng/mg total proteins) and type-II collagen contents (0.023±0.005 vs. 0.020±0.010 ng/mg total proteins) between both types of aggregates (always $P=1$).
Conclusions: The data show that FGF-2 can be overexpressed in MSCs via rAAV, promoting expansion and differentiation in a three-dimensional environment, suggesting that gene modification of MSCs via rAAV may fina application to treat articular cartilage lesions.

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CUMULATIVE EFFECT OF MECHANICAL STIMULUS ON CHONDROGENESIS OF HYMAN MESENCHYMAL STEM CELL IN SCAFFOLDS USED FOR CARTILAGE REPAIR

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Purpose: The aim of the study is to better understand the induction and remodelling of cartilage-like tissue in non-woven filamentous scaffolds intended for cartilage repair. The two objectives were to: 1) investigate the effect of cumulative mechanical stimulus on cell proliferation, morphology and metabolic activity in human bone marrow mesenchymal stem cells (BMMSCs). These cells were seeded onto non-woven pads of biomaterial and pulsatile hydrostatic pressure (PHP) applied incrementally over a 10 week period in a manner that simulates the loading conditions occurring at a cartilage repair site after the implantation of scaffolds for cartilage repair, and 2) thereby obtain an optimal post-operative rehabilitation regime following joint implant surgery.
Methods: Human BMMSCs (Lonza) from 3 donors were seeded onto 226 plasma treated polyester scaffolds (PTPS) at 2.5×10^5 cells/scaffold and divided into 2 groups; group one being subjected to PHP, group two remained unloaded and acted as control. In group one, cell-seeded